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Resource allocation across the egg laying sequence in the wild zebra finch

Simon C. Griffith[1], Emma C. Pariser[1], Barbara Tschirren[1,2], & Lee B. Astheimer[3]

[1]Department of Biological Sciences, Macquarie University, Sydney, NSW 2109, Australia

[2]Institute of Evolutionary Biology and Environmental Studies, University of Zurich, 8057 Zurich, Switzerland

[3]Centre for Integrative Ecology, Deakin University, Victoria, Australia

Corresponding Author: Simon Griffith, Department of Biological Sciences, Macquarie University, Sydney, NSW 2109, Australia

Email: simon.griffith@mq.edu.au

Phone: +61 9850 1301; fax +61 9850 9231

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Abstract

Zebra finches have played a central role in the discovery of a variety of maternal effects over the past decade, with females shown to adjust resource allocation to their eggs in response to variables such as the appearance of their partner, their own condition, and the diet on which they are maintained. In addition to being the focus of some of the most high profile individual studies that have influenced maternal effects research in birds, the multitude of zebra finch studies together provide the most comprehensive set of data to illuminate general patterns and compare different maternally derived variables. Surprisingly, to date, virtually all of this work has focused on captive populations of the zebra finch that have been domesticated for many generations, and which are typically held under relatively constant environmental and dietary conditions. Here we report the first data on resource allocation across the egg laying sequence in a free-living wild population. Reassuringly we find that the patterns that have been found in the majority of studies of domesticated populations with respect to investment across the laying sequence were all present in the wild population. The size and mass of eggs increased through the laying sequence whilst the concentration of carotenoids significantly decreased across the laying sequence. Although there was no significant pattern with respect to testosterone across the laying sequence the first two eggs had a higher level of testosterone than the last few eggs in the clutch, which is also consistent with the findings of earlier studies in captive populations.

Introduction

Studies of the zebra finch *Taeniopygia guttata* in captivity have played a pivotal role in the study of maternal effects in birds, with studies of this species often being the first to demonstrate new phenomena such as the degree of control over sex ratio (Burley 1988) and variation in the allocation of testosterone to eggs with respect to paternal quality (Gil et al. 1999). Such studies have been highly influential and played an important role in stimulating further research of a wide range of maternal effects. The captive zebra finch is a highly amenable model system for studies across a wide range of behavioural, genetic and developmental questions (Griffith & Buchanan 2010a), and it is particularly well suited to studies of maternal effects given its high reproductive rate and it's willingness to breed in laboratory conditions under intensive scrutiny. To date, the zebra finch has been the focus of a more comprehensive examination of maternal effects than other avian species by a good margin, with studies examining a range of maternally derived resources and their consequences to offspring and even grand-offspring (reviewed in Griffith & Buchanan 2010b).

The most consistent finding with respect to maternal effects in the zebra finch is the pattern of allocation by the female across the laying order in a number of important resource variables (reviewed in Griffith & Buchanan 2010b). Nearly all studies to date that have examined it have reported an increase in the size and mass of eggs across the laying sequence with the fifth egg being, on average 12.5% larger than the first (data reviewed and presented in Griffith & Buchanan 2010b). By contrast, the level of nutrients and hormones typically decrease across the laying sequence (Griffith & Buchanan 2010b). The fifth egg in a clutch contains between 39 and 50% less total carotenoids than the first one, and the level of vitamin E declines by 44-55%

(Royle *et al*. 2003; Williamson *et al*. 2006). The concentration of testosterone decreases by an average of 55% between the first and fifth egg (multiple studies reviewed in Griffith & Buchanan 2010b, but see Ward et al. 2001), while the level of oestradiol decreases by 25% in the only study to examine natural levels (Williams et al. 2005). The underlying cause of these patterns of allocation across the clutch are currently unclear, and indeed it is not even known whether they have any major functional significance to the offspring or are just a passive reflection of changing female physiology as she moves through her reproductive cycle (Groothuis & Schwabl 2008; Griffith & Buchanan 2010b). If such patterns of variation across the clutch have no significant and consistent effects on offspring development then, by definition, they will not even be maternal effects.

The relative consistency of the pattern of allocation across the laying sequence is probably an important clue to any underlying functional importance. However, an important, and largely neglected component of the variation in maternal allocation is the extent to which it is determined by environmental variation or predictability (in temperature, humidity, and nutrition). After all, maternal effects are a mechanism through which a female can alter the phenotype of her offspring to suit a particular environment (Mousseau & Fox 1998). Surprisingly, to date all of the studies of maternal effects in the zebra finch have been conducted on domesticated populations held in captivity. Furthermore, most of these study populations are held indoors (in the relatively cold and damp northern hemisphere) with fairly constant conditions of temperature, humidity and light schedules, with a relatively standard and invariant diet. Whilst such a stable captive environment is generally used for reducing the confounding effects of environmental conditions, it does not provide a good reflection of the highly variable, unpredictable and often extreme environment in which the

zebra finch evolved (Zann 1996).

It will be of great significance to a general interpretation of previous studies and a future understanding of the maternal effects they have demonstrated to know how wild females allocate resources and hormones to the individual eggs through their laying sequence. Therefore we conducted an observational study of clutches taken from free-living zebra finches breeding in their natural habitat in the desert of New South Wales, Australia.

Methods

General collection methods

Whole clutches were taken from 23 individually marked females breeding in the study population at Saloon Tank (31°03′90″S, 141°50′60″E) at Fowlers Gap Arid Zone Research Station (further details in Griffith et al. 2008). Eggs were removed (under license by NSW Parks and Wildlife Service and the Macquarie University Animal Ethics Committee) on the day that they were laid between the 5th September and 17th November 2007. We removed all clutches that were initiated during this period, in this area by different females, but only took one clutch per female. Therefore whilst the sample represents only the first part of the usual breeding period in this location, our sample should have captured most of the variation present in the population at that time with respect to female identity, age and experience, clutch size. The birds breeding at Fowlers Gap follow a seasonal pattern of reproduction with breeding generally starting in late August and declining by late December (as described in Griffith et al. 2008). The breeding activity during the year that these eggs were

sampled was quite typical of the pattern seen in this population (detailed in Griffith et al. 2008). In total 114 eggs were removed from the 23 clutches. The average clutch size was 4.96 (± 0.20 s.e.m.) with a range of three to seven eggs across the sampled clutches which reflects a random sample of those clutches laid at this location in the study population with respect to clutch size (the long-term average in this population of 4.9 (Griffith et al. 2008). The decision of which clutches to remove was also made with no consideration of parental morphology, age or breeding experience and therefore should represent a fairly random sample with respect to those variables as well. Some parents may have bred together earlier in this season or earlier in the same calendar year in different locations. Unfortunately given the high mobility of the species we were unable to determine the reproductive history of the individuals sampled. Eggs were labelled with an identity number at the time of removal and replaced with a plastic dummy egg so that the female would continue to lay her full clutch. When a female had failed to lay an egg for two consecutive mornings we considered that the clutch was complete and all of the dummy eggs were removed. Sampled eggs were measured for length and width (0.01mm) with egg volume calculated using the equation: volume = 0.519 x length x width^2 (Romanoff & Romanoff, 1949), and their mass (to the nearest 0.01grams) on the day of sampling (laying) was taken using an electronic balance.

All removed eggs were placed into artificial incubators (Brinsea Octagon 20, DX auto turn) maintained at 37.5°C with relative humidity set to 60%, for 72 hours. This standard incubation period was intended to develop the embryo to a point where the sex of developing embryo could be determined. Unfortunately these embryos were later damaged in transit and we were not able to complete the analysis of sex, and therefore account for it here.

After this period of incubation, the eggs were frozen to -20°C to await the laboratory assays of egg testosterone and carotenoids.

Analysis of yolk content

Whole yolks were removed and separated from the albumen using the different thawing rates between yolk and albumen. All egg yolks were weighed and then cut through the centre of the yolk to obtain three equivalent portions of yolk using dissection tools; each part was weighed prior to further analysis.

Testosterone assay

The wet weight of the yolk sample was taken and then the sample was homogenised using glass beads in 500 µl of commercial EIA assay buffer (Cayman Chemical, MI, USA). Each sample was spiked with 20µl of tritiated testosterone (providing approximately 2000 CPM) to allow post-extraction recovery estimates. Samples were incubated overnight at 4^{o} C. Testosterone was then extracted twice for 90 minutes in 3 ml of a 30:70 mixture of petroleum ether and diethyl ether. Ether extracts were removed after freezing the aqueous fraction in dry ice. The two extracts were combined, dried over a stream of nitrogen gas, reconstituted in 50µl ethanol, diluted 1:20 in the EIA buffer and stored at -20°C until assayed. Testosterone concentration was then measured in duplicate using Cayman EIA assay kits (#582701). All yolks from the sample clutch were run on the same plate, in total samples were run across 11 different plates (mean inter-plate variation = 25.35 %). Testosterone concentrations (ng/ml) were calculated by comparison with a standard curve. Mean extraction

recovery for samples was 61.48% with a variance of 9.49%. Of the 114 sampled eggs, we were unable to assay the concentration of testosterone in 12 eggs due to broken shells and contamination of yolk with albumen occurring during storage or processing. These failures were random with respect to clutch and egg position and do not affect our overall findings.

Carotenoid assay

The concentration of total carotenoids in the yolk was estimated using spectrometry. A portion of weighed yolk for carotenoid analysis (between 100-150 mg) was placed in a labelled 1.5ml Eppendorf tube and homogenised with acetone by sonicating the sample with a pinch of clean sand for 5 minutes. Samples were then centrifugated and the acetone phase was collected into a clean graduated tube. The yolk and sand were then re-homogenised and centrifuged with a further 2 x 1ml of acetone which was collected into the same graduated tube made up to 2ml using 100% acetone and covered with foil to protect the carotenoids from light degradation. Samples were kept on ice throughout the extraction and spectral measurement period, which was accomplished within 5 hours.

The samples were run in glass cuvettes at 4 degrees C in the range of 350-500nm and evaluated against a concentration gradient of lutein purified from marigold and supplied by Sigma-Aldrich and diluted in acetone. This method allowed us to compare the overall carotenoid spectral absorbance pattern in the samples but did not allow the identification of specific carotenoids.

Absorbance spectra were obtained from 98 yolk extracts using a UV-Visible spectrophotometer (Shimadzu UV-1601 UV-visible spectrophotometer (Shimadzu, Sydney, Australia) across the range of 350-500 nm at increments of 1nm. Yolk absorbance spectra corrected for sample mass and the area under each curve was calculated using GraphPad Prism 4 software (GraphPad Software Inc. Version 4.03).

Statistical analysis

Egg volume, egg mass, carotenoid and testosterone concentration of individual eggs were analysed using mixed effect models including a random intercept and a random slope (following Schielzeth & Forstmeier 2009) in JMP 8.0 (SAS Institute Inc., Cary, NC, 2009). Laying order was included as a fixed effect and female identity and the interaction between laying order and female identity were entered as random effects. Because of a right skew in the distributions of yolk testosterone and yolk carotenoid concentrations we log transformed these egg traits to normalise the distributions of the residuals of the statistical models.

We also present the raw data for the first and fifth egg in each clutch to enable an easier contrast to the findings from previous studies as recently reviewed (Griffith & Buchanan 2010b).

Results

Females allocated progressively more overall in the eggs laid later in the clutch with a significant increase in both the egg mass ($b = 0.017 \pm 0.005$, $F_{1,25.23} = 11.97$, $p = 0.002$, $N = 23$ clutches; Figure 1a), and the egg volume (slope ± 1S.E: $b = 16.19 \pm$

4.68, $F_{1,23.56} = 11.94$, $p = 0.002$, $N = 23$ clutches; Figure 1b), across the laying sequence. On average the fifth egg was 9% larger (by volume) and 10% heavier than the first egg to be laid.

In contrast to the overall size of the eggs, females significantly reduced the concentration of carotenoids through the laying sequence ($b = –0.114 \pm 0.045$, $F_{1,8.22} = 6.44$, $p = 0.034$, $N = 21$ clutches; Figure 1c), with the fifth egg having an average concentration that was 35% lower than the first egg laid. There was no significant difference in the concentration of yolk testosterone across the laying sequence ($b = –0.044 \pm 0.033$, $F_{1,17.61} = 1.72$, $p = 0.206$, $N = 22$ clutches). The average concentration of testosterone in the fourth and fifth eggs across the sample was 22%, and 12% lower (respectively) than the level in the first egg (Figure 1d). Female identity accounted for a large proportion of variance in egg composition whereas the proportion of variance explained by the interaction between female identity and laying order was generally small (Table 1). It indicates that there are large differences in egg investment between females, but little differences in the change in egg investment with laying order between females.

There was a strong positive correlation between the egg volume and egg mass (Pearson correlation: $r = 0.952$, $p < 0.001$, $N = 114$). No other egg traits were significantly correlated (all $p > 0.137$).

Discussion

We found that in a population of wild zebra finches, breeding in the Australian arid zone, females allocate resources to eggs differently across the laying sequence. Eggs

increase progressively in size and mass whilst the concentration of carotenoids in the yolk decreases significantly through the laying sequence, and the patterns occur consistently across all females. All of these patterns of allocation are consistent with many previous findings demonstrated in a number of populations of domesticated zebra finch studied in captivity in the northern hemisphere. Our findings therefore suggest that the previous work conducted on these aspects of maternal investment is reflective of the patterns seen in wild birds breeding in their native environment and operating under a natural set of environmental and ecological conditions. This validation of the work conducted in captivity is important due to the prominence of studies of this important model system in the developing field of maternal effects in birds (reviewed in Griffith & Buchanan 2010b).

The only variable on which our findings did not appear to support the previously demonstrated pattern of investment in laboratory birds was the concentration of testosterone in the yolk. The allocation of testosterone did not vary significantly across the laying sequence in our sample of 22 clutches. However, we believe that it would be wrong to conclude that this is inconsistent with earlier findings in the laboratory (e.g. Gil et al. 1999; 2004; Gilbert et al. 2005; 2007; Rutstein et al. 2004a). Our sample size of clutches was relatively low and the power of our test would be relatively low to determine that there is no allocation across the laying sequence. The data presented in Figure 1d indicates a potential pattern that would be consistent with previous demonstrations of a decreasing concentration across the laying sequence, and indeed the average concentration in the fourth and fifth eggs in our sample was between 12-22% lower than the level in the first egg laid. Previous studies of captive birds have found average decreases of between 21-33% between the first and fifth egg (data reviewed in Griffith & Buchanan 2010b),

however in subsets of data from at least two previous studies the general pattern (of a declining concentration) was not observed (Rutstein et al. 2004a; Gilbert et al. 2005).

There are two known issues that undermine the ability to determine as clear a pattern with respect to testosterone as other egg variables such as carotenoids or size. The first is that testosterone is believed to have sex-specific effects on embryos (e.g. von Engelhardt et al. 2006), and any straightforward pattern of allocation across the laying sequence may be somewhat obscured by differential allocation of testosterone with respect to the sex of embryo contained within each egg (Rutstein et al. 2004a; Gilbert et al. 2005; Rutkowska & Cichón 2002). In this study we had intended to determine the sex of the embryos and therefore incubated each egg for 72 hours before sampling them. Unfortunately the sampled embryos were damaged in transit and molecular sex determination was not possible for this sample. Our treatment of the eggs after collection however does relate to the second issue which is that embryos will probably synthesise their own steroids before hatching (Ottinger & Abdelnabi 1997), or metabolise those deposited by the mother (Bruggeman et al. 2002), and therefore it is possible that the level of testosterone after 72 hours of incubation is not the same as that deposited by the female at the time of egg laying. However, the study by Gilbert et al. (2007) of domesticated zebra finches found that there was no change in the level of testosterone in the first three days of incubation and also no sex-specific uptake or metabolism of hormones during this same period. Therefore, it seems reasonable to assume that our measures of testosterone in these eggs removed from a natural population and incubated for 72 hours, are representative of the level of initial maternal hormonal deposition.

Being wholly observational in nature, our study is unable to shed any new insight on the function of the intra-clutch variation in size, mass and nutrient contents.

Furthermore, our findings are only representative of a single site and year of sampling, which we believe to be fairly typical of that experienced by zebra finches breeding in the arid zone (see Griffith et al. 2008). However, it is quite possible that different patterns may emerge in more extreme situations, for example when birds are breeding in ecological conditions when food is either more constrained or much more abundant. However, we believe that, as a starting point, our demonstration of these egg order effects in a natural population is of great relevance to future work. Firstly, it will provide a foundation for future experimental work in wild populations and perhaps most importantly it provides validation for the further intensive study of these effects, and their functional outcomes, in captive populations. The captive domesticated zebra finch has already proved to be a very important model system for the study of numerous behavioural, developmental and physiological traits (Griffith & Buchanan 2010a). The demonstration that the most consistently observed maternal allocations in domestic populations are also present in truly wild populations under normal ecological conditions will no doubt prove reassuring to future studies that seek to explain the functional relevance of these subtle but significant investment allocations that females make across their clutch.

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375 **Figure Legends**

Figure 1. The average level of maternal investment (± s.e.m.) across the laying sequence (eggs 1 to 5) with respect to a) mass, b) volume, c) carotenoids, d) testosterone. The sample sizes are given above each column on the graph.

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Table 1. Variance in egg composition explained by female identity and the interaction between female identity and laying order.

Egg trait	Variance explained by female identity	Variance explained by female identity x laying order
Egg volume	41.2%	0.9%
Egg mass	39.4%	1.0%
Yolk carotenoids	29.5%	0.5%
Yolk testosterone	22.3%	6.5%

385

Figures

Figure 1a

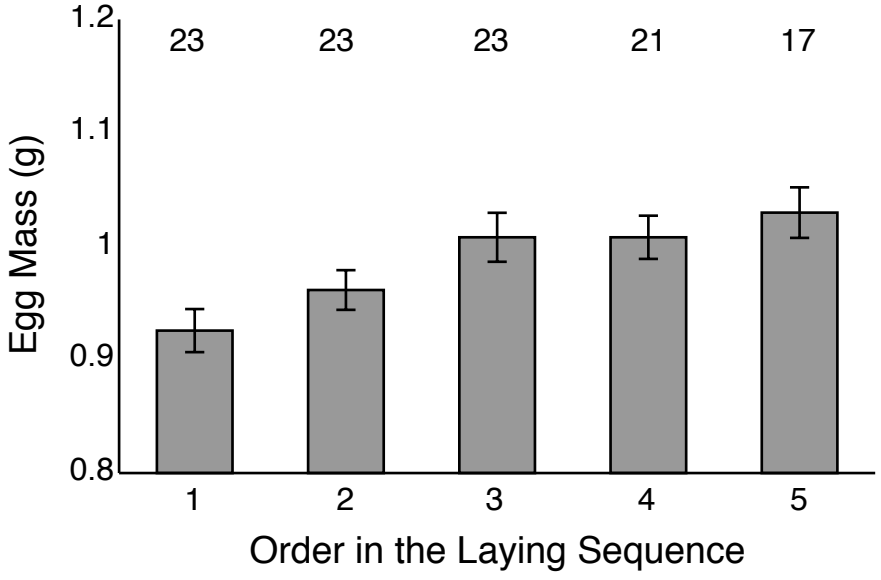

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Figure 1b

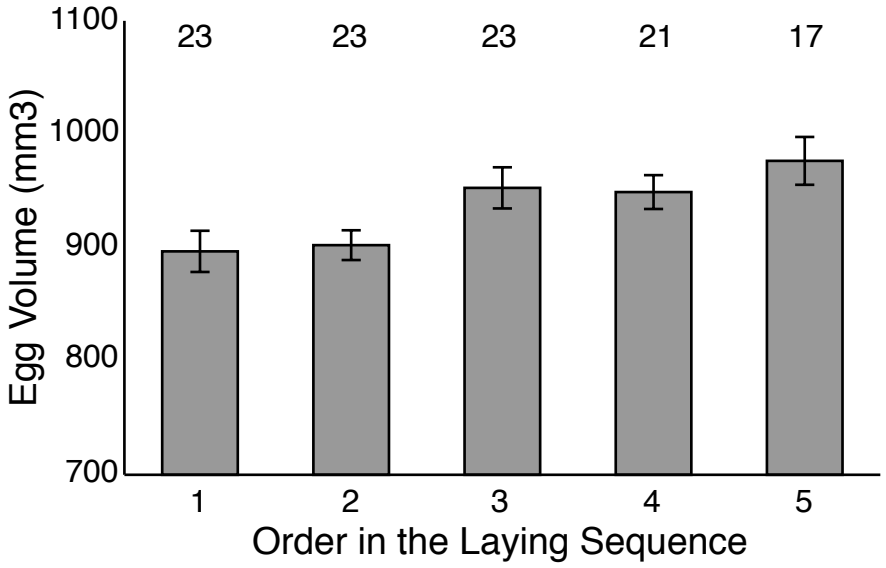

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Figure 1c

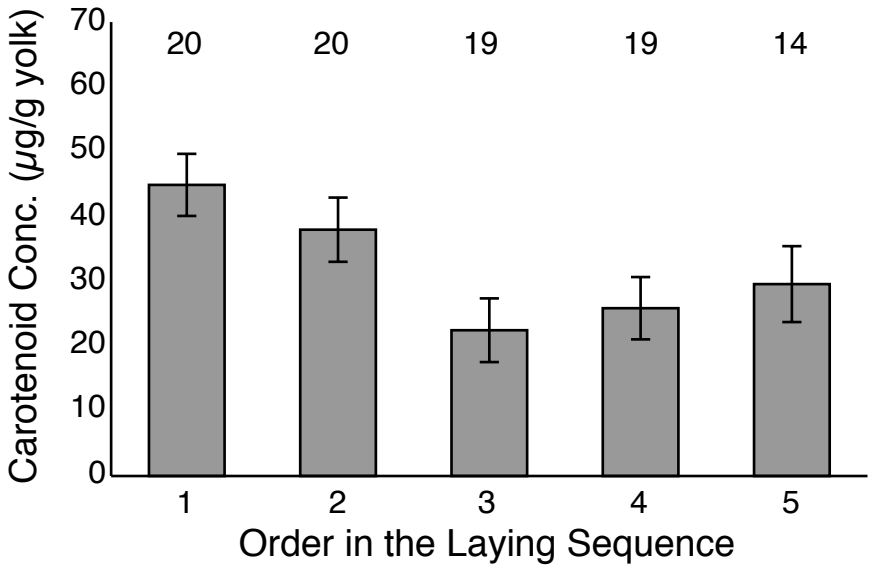

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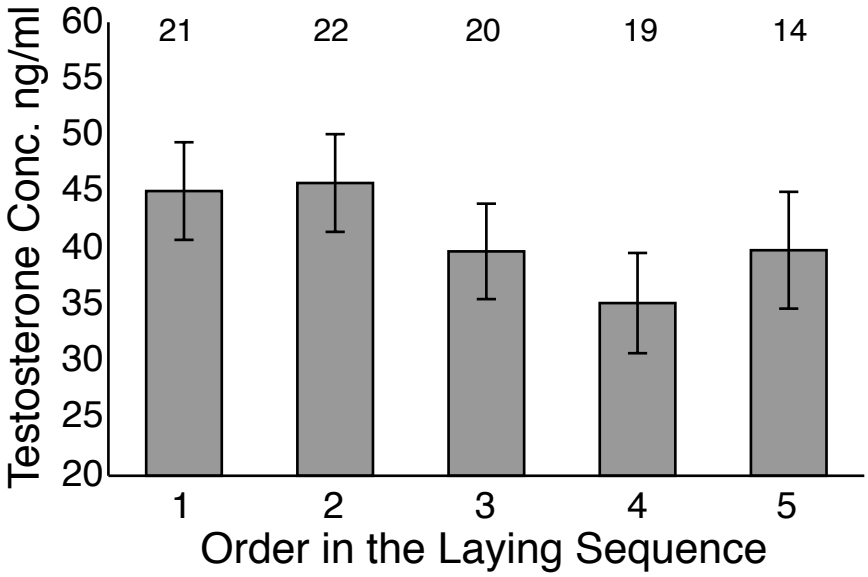
Figure 1d



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